Estimation of Hymenopteran Parasitism in Cereal Aphids by Using Molecular Markers

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ABSTRACT Polymerase chain reaction (PCR) primers were designed and tested for identification of immature parasitoids in small grain cereal aphids and for estimation of parasitism rates. PCR technique was evaluated for 1) greenhouse-reared greenbugs, *Schizaphis graminum* (Rondani), parasitized by *Lysiphlebus testaceipes* Cresson and 2) aphids collected from winter wheat fields in Caddo County, Oklahoma. For greenhouse samples, parasitism frequencies for greenbugs examined by PCR at 0, 24, and 48 h after removal of *L. testaceipes* parasitoids were compared with parasitism frequencies as determined by greenbug dissection. PCR was unable to detect parasitism in greenbugs at 0 and 24 h postparasitism, but it was able to detect parasitoids 48 h after parasitoid removal at frequencies that were not significantly different from dissected samples. Field-collected samples were analyzed by rearing 25 aphids from each sample and by comparing parasitoid frequencies of mummies developed and PCR performed on another 50 aphids. Aphid samples included corn leaf aphids, *Rhopalosiphum maidis* (Fitch); bird cherry-oat aphids, *Rhopalosiphum padi* (L.); English grain aphids, *Sitobion avenae* (F.); and greenbugs. Mummies were isolated until adult emergence, whereupon each parasitoid was identified to species (*L. testaceipes* was the only parasitoid species found). Parasitism detection frequencies for PCR also were not statistically different from parasitism frequencies of reared aphids. These results indicate that PCR is a useful tool for providing accurate estimates of parasitism rates and especially for identification of immature parasitoids to species.

KEY WORDS *Lysiphlebus testaceipes*, *Schizaphis graminum*, PCR, parasitoid eggs

SMALL GRAIN APHIDS ARE attacked by several parasitoid species (Royer et al. 1998). *Lysiphlebus testaceipes* (Cresson), (Hymenoptera: Aphidiidae) is arguably the most important parasitoid of small grain aphids throughout the central and western wheat-growing regions of the United States (Jackson et al. 1970, Walker et al. 1973, Archer et al. 1974, Summy et al. 1979, Rice and Wilde 1988, Giles et al. 2003). This oligophagous parasitoid frequently suppresses greenbug, *Schizaphis graminum* (Rondani) (Homoptera: Aphididae), and other small grain aphid populations below economic thresholds on crops such as wheat and grain sorghum in the southern Great Plains (Patrick and Boring 1990, Jones 2001, Giles et al. 2003).

Effective use of parasitoids for control of small grain aphids requires knowledge of species composition and valid estimates of the rate of attack (Patrick and Boring 1990, Royer et al. 1998). Identification of these aphid parasitoids by use of taxonomic keys is often difficult. Frequently, observable differences between species consist of little more than a wing vein that is slightly longer in one species than another closely related species, such as *Aphidius colemani* Viereck versus *L. testaceipes* (Pike et al. 1997, Atanassova et al. 1998). Although it is relatively simple to find aphid mummies because they differ in appearance from their aphid host, detection of parasitoids in nonmummified aphids is time-consuming, requiring rearing of live specimens until the adult parasitoid emerges (Royer et al. 1998). Dissection of aphid hosts is useful to confirm attack by parasitoids (Jones et al. 2003), but identification of the immature parasitoid to species is virtually impossible. A potential solution to each of these problems is the use of polymerase chain reaction (PCR).

Polymerase chain reaction results in the rapid production of multiple copies of a specific nucleotide sequence found in DNA (Mullis 1990). The PCR technique makes it possible to analyze nucleotide sequences in samples that contain amounts of DNA that are either too small, or too degraded, to permit other types of nucleic acid analysis (Glick and Pasternak 1998). This feature of PCR has made possible the development of experimental and diagnostic molecular biology techniques, which were previously ex-

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tremely time-consuming or impossible to perform. For example, Ward et al. (1990) successfully used PCR to diagnose an arboviral infection (Dugbe viral RNA) in an arthropod vector. By using PCR, diagnoses of viral infection were obtained within 48 h, whereas biological assays took at least 8 d to complete. Another feature of PCR is that live specimens are not required (Persing et al. 1990). Processing samples can be performed at the researcher's leisure because samples can be kept for extended periods in the freezer (Glick and Pasternak 1998).

We chose to use the 16S rDNA gene in our research. The 16S rDNA gene fragment is of interest because little variation in the coding regions is found within species and could be used as a marker indicating the presence or absence of parasitoids in a host. Additionally, the 16S rDNA gene occurs as 100 or more copies within each cell compared with single-copied nuclear genes (Dover 1982, 1986). Finally, Chen et al. (2002) already sequenced the 16S rDNA gene for all of the common small grain aphid parasitoids found in the southern Great Plains.

Our objectives for this research were to 1) determine accuracy of PCR primers designated LtepuF and LtepuF for detection of *L. testaceipes* eggs and larvae inside greenbugs at 0, 24, and 48 versus 72 h postoviposition when dissection can provide reliable parasitoid detection (Jones et al. 2003) in a controlled laboratory experiment; and 2) further test the accuracy of these primers for detection of *L. testaceipes* eggs and larvae inside aphids collected from winter wheat fields in Oklahoma.

Materials and Methods

Greenbug and Parasitoid Colonies. Biotype "E" greenbugs were obtained from colonies maintained at the USDA–ARS Plant Science and Water Conservation Research Laboratory at Stillwater, OK, and established on grain sorghum ('SG-925') grown in a fritted clay and sphagnum moss mixture. Insect colonies and all sorghum plants were kept inside double-walled fine mesh cages within a greenhouse (≈22–28°C). The double-walled cages prevented contamination of colonies by feral greenbugs and parasitoids while permitting ample airflow (Jones et al. 2003). Fresh plants were rotated as needed into cages housing colonies.

L. testaceipes was isolated from specimens collected at Perkins, OK, in fall 1999 (40 *L. testaceipes* adults isolated from ≈50 greenbug mummies). Pots of sorghum infested by greenbugs were placed in the colonies every 3–4 d to maintain a steady supply of parasitoids. Parasitoid colonies were maintained at 22 ± 0.5°C and a photoperiod of 12:12 (L:D) in double-walled fine mesh cages kept in a growth chamber (Jones et al. 2003).

Primer Design. Primers were designed using 16S rDNA sequences that represent commonly found cereal aphids and their parasitoids. The aphids consisted of corn leaf aphid, *Rhopalosiphum maidis* (Fitch); bird cherry-oat aphid, *Rhopalosiphum padi* (L.); Russian wheat aphid, *Diuraphis noxia* (Mordvilko); yellow sugarcane aphid, *Sipha flava* (Forbes); English grain aphid, *Sitobion avenae* (F.); and greenbug (Y.C., unpublished data; GenBank accession nos. AY745772–AY745781). Parasitoids consisted of *Aphelinus asychis* Walker, *Aphelinus albipodus* Hayat & Fatima, *Aphelinus varipes* (Foerster), *Aphelinus hordei* (Kurdjumov), *Aphytis melinus* DeBach, *Diaeretiella rapae* (M'Intosh), *Aphidius colemani*, *A. ervi* Haliday, *Aphelinus matricariae* Haliday, and *L. testaceipes* (Chen et al. 2002; GenBank accession nos. AF289130–AF289148). We used GCG Wisconsin Package UNIX version 10 (Genetics Computer Group, Madison, WI) for alignment and analysis. The single-base detection technique (Kwok et al. 1990) was used to design primers.

A forward and a reverse primer were designed on the conserved part of the 16S rDNA gene to detect the general presence of a parasitic wasp. These general primers were designated WaspF1 (5′-ACC TG TTTAT CAAAA ACATG-3′) and WaspR (5′-CGAGG TCGCA ATCTT TTTTA-3′). A 465-bp PCR product was produced by using of WaspF1 and WaspR primer pair. Development of these general primers was necessary because field-collected aphids could harbor any of the listed parasitoids.

Specific primers were then developed on the conserved part of the 16S rDNA gene as well, to detect the presence of *L. testaceipes* DNA from any population, yet only amplify 16S rDNA from *L. testaceipes* (Chen et al. 2002). These primers were designated LtepuF (5′-CAAAA ACATG TCTTA TTGAA-3′) and LtepuR (5′-CCCCA ATTAA ATATT AGTTT AA-3′). The LtepuF and LtepuR primers produced a 299-bp PCR product.

Laboratory Study. Approximately 500 second to third instars of greenbugs were placed on each of three pots of grain sorghum and allowed to settle for 4 h. Each sorghum plant was caged such that greenbugs were excluded from the soil, yet they were exposed to ample airflow. Twenty-five female *L. testaceipes* parasitoids from laboratory colonies were introduced into each cage and allowed to parasitize greenbugs for 4 h, after which they were immediately removed by aspiration. Four groups of 100 greenbugs were removed randomly from each plant for analysis, at 0, 24, 48, and 72 h postparasitoid removal. The first three groups of 100 greenbugs from each plant were each placed in 90% ethanol and frozen until PCR was performed. The last group removed at 72 h was placed in 2% saline and dissected to detect presence of *L. testaceipes* larvae. Dissections were only performed on greenbugs removed at 72 h because parasitoid eggs are difficult to detect, thus delaying dissections until after parasitoid eggs hatch greatly improved accuracy of the data (Hofsvang and Hågvar 1978, van Steenis 1993). This procedure was replicated three times.

Greenbugs destined for PCR analysis were placed individually in 1.5-ml microcentrifuge tubes. They were then homogenized for 1 min with a pestle mounted in a battery-powered drill in 80 μl of isolation buffer containing 0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl (pH 9.1), 0.05 M EDTA, 1% SDS, and

20 μg/ml RNAase A. The homogenate was vortexed briefly and incubated for 30 min in a 65°C water bath. The sample was then spun in a centrifuge for 3 min. The supernatant fluid was removed and placed in a new tube. Five microliters of 3 M sodium acetate and 110 μl of ice cold 100% ethanol were then added. This solution was mixed well and then centrifuged for 30 min to collect the precipitated nucleic acid salts at the bottom of the tube. The salts were air-dried and then resuspended in 50 μl of distilled water.

One microliter of the nucleic acid salt solution was placed in a new 0.5-μl vial along with enzyme storage buffer A consisting of 50 mM Tris-HCl (pH 8.0 at 25°C), 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 50% glycerol, and Triton X-100, along with $MgCl_2$, *Taq*DNA polymerase, NTPs (all supplied by Promega, Madison WI), primers LtepuF and LtepuR for *L. testaceipes*, and distilled water. Each vial was placed in a PTC-100 thermocycler (MJ Research, Watertown, MA). DNA was denatured for 3 min at 94°C, followed by 35 amplification cycles, with 30-s denaturing at 94°C, 30-s annealing at 55°C, and 1-min extension at 72°C. DNA was finally extended for 1 min at 72°C after amplification. After amplification, PCR products were separated on a 1.5% agarose electrophoresis gel, stained with ethidium bromide, and photographed under UV light. Presence of parasitoid DNA is confirmed by the migration of a band of DNA down its respective lane that matches the band in the known DNA lane. Comparisons were made using PROC MIXED (SAS Institute 1999) between samples where the parasitism rate was measured by dissection 0, 24, and 48 h postparasitism PCR.

Field Study. Approximately 75–100 aphids were collected from each of four wheat fields in Caddo County, OK, during November 2001. For each group of aphids collected, 25 randomly selected aphids were isolated and reared on wheat plants for up to 7 d until mummies developed. Each mummy was then isolated in a 1.5-μl microcentrifuge tube, allowed to emerge, and identified to species by taxonomic key (Pike et al. 1997). Another 50 aphids were randomly selected from each group of collected aphids and were frozen in 90% ethanol to preserve until PCR amplification was performed. Because of the possibility of other parasitoid species being present in the field-collected samples, we used the general parasitoid primers WaspF1 and WaspR to determine the presence of any parasitoid DNA. This PCR procedure used differed from the laboratory experiment by the primers used and the thermocycler program in which DNA was denatured for 3 min at 94°C, followed by 35 amplification cycles, with 30-s denaturing at 94°C, 30-s annealing at 51°C, and 1-min extension at 72°C. DNA was finally extended for 1 min at 72°C after amplification. If a parasitoid was detected in the sample by using general parasitoid primers, the sample was then reexamined using LtepuF and LtepuR primers to discover whether *L. testaceipes* was present. Comparisons were then made using PROC MIXED between samples where the parasitism rate was measured by rearing aphids and those samples where the parasitism rate was measured by PCR.

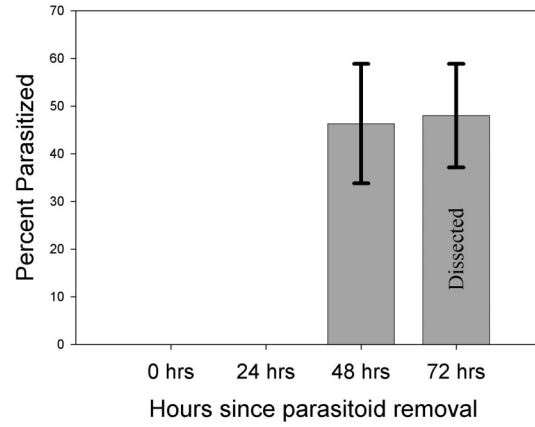

Fig. 1. Mean parasitism rates in laboratory prepared greenbug specimens as determined by PCR detection of parasitoid DNA in greenbugs at 0, 24, and 48 h after removal of *L. testaceipes* females compared with parasitism rate determined by dissection of greenbugs at 72 h after removal of parasitoids (48 versus 72 h: $t = 0.18$, df = 6, $P = 0.86$, SAS PROC MIXED).

Voucher Specimens. Voucher specimens of aphid species have been deposited in the Department of Entomology and Plant Pathology Museum at Oklahoma State University, Stillwater, OK. Voucher specimens of parasitoid species were reported previously (Chen et al. 2002).

Results and Discussion

We were unable to detect parasitism in greenbugs in the 0 and 24 h postparasitoid removal samples with PCR in the laboratory study (Fig. 1). However, we were able to detect *L. testaceipes* in 48 h postparasitoid removal samples at frequencies not significantly different from dissected samples ($t = 0.18$, df = 6, $P = 0.86$). In field-collected samples consisting of corn leaf aphids, bird cherry-oat aphids, greenbugs, and English grain aphids, samples that tested positive for parasitoid presence (WaspF1 and WaspR primers) also tested positive for *L. testaceipes* (LtepuF and LtepuR primers) in every instance. Previous research shows that this is not unusual during late fall and winter in the southern Great Plains (Jones 2001, Giles et al. 2003). Samples reared to parasitoid emergence also were only parasitized by *L. testaceipes.* Statistical analysis revealed that the percentage of hosts parasitized by *L. testaceipes* as detected by PCR was not statistically different from parasitism percentage as determined by rearing aphids (Fig. 2; $F = 1.85$, df = 3, $P = 0.27$, PROC MIXED).

PCR techniques are able to detect and amplify DNA fragments from very minute quantities (Johner et al. 1999, Zhu and Greenstone 1999). Using PCR, they were able to detect very small amounts of polydnavirus DNA in the parasitized egg of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) shortly

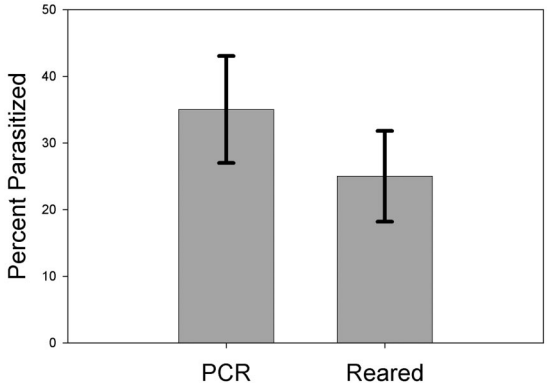

Fig. 2. Mean parasitism rate determined by PCR detection of parasitoid DNA (using LtepuF and LtepuR primers) in samples of aphids collected in winter wheat fields compared with parasitism rate determined by key identification of adult parasitoids that emerge from corresponding samples of aphids reared for 7 d ($F = 1.85$, df = 3, $P = 0.27$, SAS PROC MIXED).

after oviposition by the parasitoid *Chelonus inanitus* (L.) (Hymenoptera: Braconidae). PCR should be able to amplify even one copy of the target gene. However, it is more likely that multiple copies are needed to provide a large enough titer of DNA for amplification (Glick and Pasternak 1998).

These results demonstrate that it is possible to reliably use PCR to detect and identify the DNA of insects parasitizing small grain aphids. However, it does have its limitations. We were unable to detect parasitoid DNA at 0 and 24 h postparasitoid removal, but we were very successful at 48 h postparasitoid removal. These detection times differ somewhat from work done by Zhu and Greenstone (1999), who were able to detect cereal aphid parasitism by *A. asychis* by PCR. Additionally, Ratcliffe et al. (2002) were able to differentially detect larvae of four parasitoid species in the genus *Muscidifurax* (Hymenoptera: Pteromalidae) within 24 h after they parasitized stable fly, *Stomoxys calcitrans* (L.), pupae.

A possible reason for the lag time necessary for PCR to detect aphid parasitoids might be that the DNA of *L. testaceipes* is "trapped" inside a tough, yet flexible egg chorion that is able to resist our attempts to mechanically penetrate and release the DNA. Parasitoids such as *L. testaceipes* are typically alecithal (yolk poor) and expand greatly in size after deposition in the host. Their embryonic development relies upon immersion in the host's hemolymph and uptake of host nutrients (Jervis and Copland 1996). It takes ≈2 d after a greenbug is parasitized before a *L. testaceipes* egg hatches (Hardee et al. 1990, Knutson et al. 1993). If disruption of the egg chorion were indeed necessary for PCR to detect *L. testaceipes*, perhaps a chemical means of penetrating the egg would lessen the time necessary for detection.

At present, this technique is only being used to detect a primary parasitoid, namely, *L. testaceipes.* A complex of obligate primary parasitoids, facultative primary parasitoids and hyperparasitoids attacks cereal grain aphids (Pike et al. 1997, Royer et al. 1998). As long as parasitoid DNA is present in the host, PCR should be able to detect its presence. At present, there is no definitive method to determine which parasitoid would "win" the competition for the host aphid resources should there be more than one species of parasitoid present. Additionally, PCR cannot determine whether the host aphid was parasitized multiple times by the same parasitoid species.

Detection of parasitoid larvae within their hosts and identification of the parasitoid species by PCR will be very useful for researchers to examine aphid samples postmortem and derive useful data from them. Taxonomic keys are available to identify adult aphid parasitoids to species (Pike et al. 1997, Atanassova et al. 1998). This necessitates live specimens and timely inspection of those samples. Use of PCR could make handling of aphid samples easier, eliminating the need for host plant production and daily observations required for rearing procedures. Additionally PCR could be used to "resurrect" previously unusable samples where the aphid host died before the parasitoid could develop into an adult. Archived specimens taken long ago also could be reexamined to determine whether they were parasitized and, with the proper primers, determine what species of parasitoid was responsible.

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